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CITATION:

Hisazumi, Haruo. STUDIES ON THE FIBRINOLYTIC ACTIVITY IN CANCEROUS BLADDER
TISSUES : A STUDY BY GEL FILTRATION. 泌尿器科紀要 1975, 21(8): 731-734

ISSUE DATE:

1975-08

URL:

<http://hdl.handle.net/2433/121866>

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STUDIES ON THE FIBRINOLYTIC ACTIVITY IN CANCEROUS BLADDER TISSUES: A STUDY BY GEL FILTRATION

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Abstract: The extract from cancerous bladder tissue was fibrinolytically inactive in fibrin plate assay and contained approximately 250 units per ml of urokinase inhibitor. By means of gel filtration (Sephadex G-200) the activators and inhibitors of fibrinolysis contained in the extract were separated. Tissue activator partially purified from a normal human bladder was inhibited by cancerous bladder tissue extract. To a much greater extent urokinase was inhibited by the same extract, specifically, 30 Ploug units of urokinase were almost completely inhibited by the 250 units of urokinase inhibitor present in the extract, whereas only 30 per cent of the 8 Ploug units of tissue activator was inhibited by the same amount of extract. The normal bladder tissue extract showed moderate fibrinolytic activity caused by an activator of plasminogen and very slight inhibitory activity (approximately 6 units per ml) against urokinase.

The presence of an inhibitor against urokinase (urinary plasminogen activator) in the blood and tissues has been reported in connection with pregnancy¹⁻⁵, and it has been strongly suggested that because the placenta and amniotic fluid contain a higher concentration of urokinase inhibitor this plays a role to the diminution in plasma fibrinolytic activity in pregnant women^{4,5}. In a previous paper⁶, the authors demonstrated that cancerous tissue fragments of the bladder or its extracts showed a marked inhibitory effect upon urokinase and tissue plasminogen activator, especially selective for urokinase. Recently, Bernik and Kwaan⁷ reported that cells from the adult human bladder in culture yielded an urokinase type activator in immunoassays and that its activity was hindered by inhibitory activity directed selectively against urokinase, and that the inhibitory activity was accumulated in the supernatant of the culture. Their data

have strongly suggested that the urokinase inhibitor plays a part in maintaining the antagonistic balance of the fibrinolytic system in tissues.

The aim of this present study was to investigate the relationship between the inhibition of urokinase and tissue activator by the extract from cancerous tissue of the bladder, and to segregate activators and inhibitors of fibrinolysis from the extract by means of gel filtration (Sephadex G-200).

MATERIALS AND METHODS

Tissues of the bladder: Cancerous tissue of the bladder was obtained from a fresh operation specimen available in the case of a man who had undergone total cystectomy for transitional cell carcinoma of the bladder; Grade 3, Stage C and solid carcinoma. The normal bladder tissue was surgically taken from a patient undergoing prostatectomy as a control. The tissues were either examined immediately or kept at minus 20°C until they were used.

Fibrinogen: Bovine plasminogen-rich fibrinogen (Armour Pharmaceutical Co.

This investigation was supported, in part, by a grant (No. 757181) for Miscellaneous Scientific Research from the Education Ministry.

Kankakee, Ill.) was dissolved in borate-saline buffer (pH 7.75, ionic strength 0.15) at a concentration of 0.2 per cent, and used for fibrin plates.

Thrombin: Topical thrombin (Park, Davis and Co., Mich.) was dissolved in 0.9 per cent sodium chloride at a concentration of 50 NIH units per ml and stored at minus 20°C.

Urokinase: Urokinase (Green Cross Corp., Osaka) was dissolved in borate-saline buffer. A stock solution was made at a concentration of 500 Ploug units* per ml, and stored at minus 20°C.

Tissue activator: Tissue activator was prepared from the human bladder by the method of Astrup and Sterndorff⁹. The fresh bladder was obtained through the courtesy of the Department of Pathology, Kanazawa University. The lyophilized powder obtained was dissolved in borate-saline buffer in a concentration of 40 mg per ml, which showed the fibrinolytic activity corresponding to 8 Ploug units of urokinase in the fibrin plate assay.

Tissue extract: After fat and blood coagula were removed, the tissues were minced, and homogenized with a 10-fold volume of cold borate-saline buffer in a wet grinder for 10 minutes at 4°C, and then centrifuged for 30 minutes at 23,000× G. Tissue extracts were obtained as the supernatant.

Gel filtration (Sephadex G-200): This procedure was performed as previously described⁹. Ten ml of the extract was layered between the gel bed and buffer solution. The elution was carried out at a flow rate of 5 ml per hour and the effluent was collected in 5 ml portions in a fraction collector operating on a volume basis at approximately 4°C.

Fibrinolytic assay: Fibrin plates, the standard and heated one, were made by the methods of Astrup and Müllertz¹⁰ and Lassen¹¹. Thirty microliters of the extracts or fractions were applied on fibrin plates

and incubated for 18 hours at 37°C. The fibrinolytic activity was recorded on the diameter products in mm² of the lysis zones.

Assay of inhibitory activity: With fibrin plates, the inhibitory activities of the extract against urokinase and tissue activator were determined according to the method of Kawano, Morimoto and Uemura⁴. As a 100 per cent activator solution, 30 Ploug units per ml of urokinase and 40 mg per ml of tissue activator were used. To 0.5 ml of 100 per cent activator solution equal volumes of the extract were added at various concentrations. Then thirty microliters of this mixture were dropped on a fibrin plate in triplicate and the lysis areas were measured after 18 hours at 37°C. Separately, each of 100 per cent, 50 per cent or 25 per cent activator solutions was mixed with an equal volume of phosphate buffer (pH 7.8) and standard curves depicting lysis areas versus activator concentrations were prepared for urokinase and tissue activator. Remaining percentages of the activator activities when mixed with extract were read from the corresponding lysis areas of the standard curves. On the other hand, one inhibitory unit was defined as the activity necessary to inhibit one Ploug unit of urokinase, and so the number of inhibitory units per ml of the extract was calculated by the procedure of Kawano, Morimoto and Uemura⁴. In the case of the fractions, as 100 per cent activator solution 2 Ploug units per ml of urokinase and 10 mg per ml of tissue activator were employed, and inhibition percentages of the activator activities when mixed with the fractions were calculated from the standard curves.

RESULTS

Inhibition of urokinase and tissue activator by the extract: Urokinase inhibitory activity (urokinase inhibitor) of the extract obtained from the cancerous tissue was 250 inhibitory units per ml. As shown in Fig. 2, 30 Ploug units per ml of urokinase were almost completely neutralized at a urokinase inhibitor concentration of 250 units per ml. Thirty per cent of the tissue activator activity (8 units per ml) was neutralized

* Ploug unit: One Ploug unit, in terms of fibrinolytic assay, is approximately equivalent to 1.4 CTA units, 3 Abott units or 17 Sterling-Winthrop units (Refer to S. Sherry et al.: J. Lab. & Clin. Med., 64: 145~153, 1964).

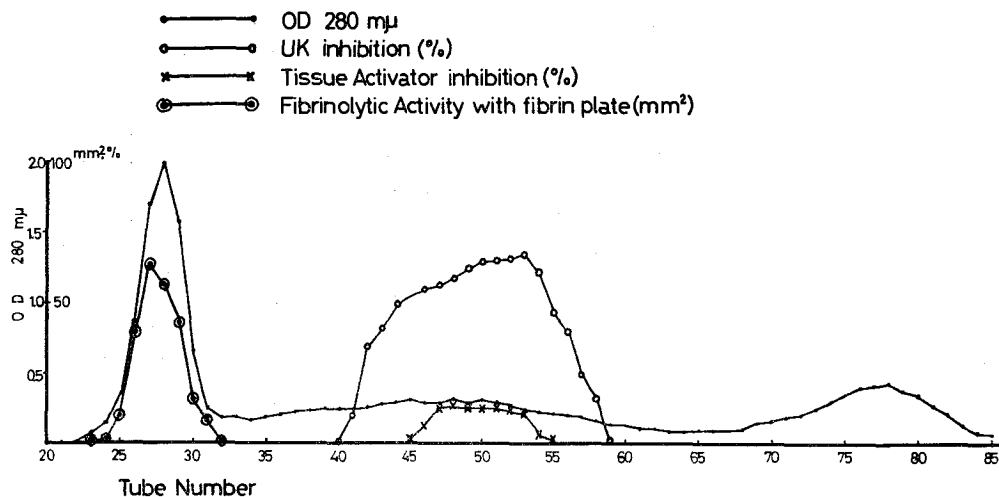


Fig. 1. Elution pattern of cancerous tissue extract of the bladder (Sephadex G-200).

at this urokinase inhibitor concentration. As the relationship between the units of urokinase inhibitor and the inhibition percentages was that of a sigmoid curve, an approximately 8 times higher concentration of urokinase inhibitor was necessary to completely neutralize 30 Ploug units per ml of urokinase.

In the case of the extract obtained from the normal bladder tissue, its urokinase inhibitor concentration was only about 6 inhibitory units per ml and it showed no inhibitory activity against tissue activator. On the other hand, its fibrinolytic activity

was approximately 200 mm² corresponding to approximately 5 Ploug units of urokinase in fibrin plate assay.

Gel filtration of the tissue extract: Shown in Fig. 1 is the elution pattern of the cancerous tissue extract which had shown no fibrinolytic activity in fibrin plate assay. The protein determination was made by measuring the optical density at 280 mμ. The first protein peak, representing the major part, was fibrinolytically active on the standard fibrin plates. No fibrinolytic activity was recorded on the heated plasminogen-free fibrin plates, therefore, this result indicated that the activity was caused by an activator of plasminogen. The inhibitory activities of the fractions against urokinase and tissue activator appeared in the part of another much smaller peak in the protein distribution. This peak was followed by an additional peak of inactive material. The highest inhibitory level toward the urokinase activity (2 Ploug units per ml) in the fractions was approximately 68 per cent inhibition, while that toward the tissue activator activity (10 mg per ml) was approximately 13 per cent inhibition.

The fractions from the normal tissue extract showed very slight fibrinolytic activity in the part of the first peak of protein distribution, but no inhibitory activity in any fractions, because the extract used contained very small amounts of fibrinolysis inhibitors and they were

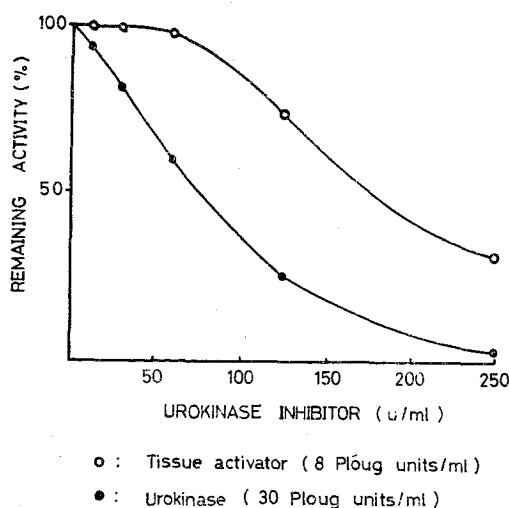


Fig. 2. Inhibition of urokinase and tissue activator by cancerous tissue extract of the bladder (fibrin plate method).

extremely diluted with the effluent in the procedure of gel filtration.

DISCUSSION

The content of urokinase inhibitor in the extract from cancerous tissue used was very high as compared with that of normal tissue of the bladder. However, its content was considered to be slight in comparison with that of the placental extract reported by Kawano, Morimoto and Uemura⁴⁾. The inhibitory effect of the cancerous tissue extract upon urokinase and tissue activator was similar to that of urokinase inhibitor purified from the placental extract, that is, urokinase was selectively inhibited by the cancerous extract, while tissue activator was slightly inhibited. These results indicate that the tissue fibrinolytic system comprises at least two antagonistic relations, and suggest that the cancerous tissue is strikingly on the side of the inhibitor as compared with the normal tissue. Moreover, the present paper indicates that activators and inhibitors of fibrinolysis contained in a supernatant of cancerous bladder tissue may be distinguished following separation by gel filtration.

To clarify the details of tissue fibrinolytic systems, further studies are necessary.

ACKNOWLEDGEMENT

Grateful acknowledgement is made to Prof. K. Kuroda for his constant interest and guidance in this investigation.

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(Accepted 1975. 6. 6.)

ゲル濾過法による膀胱癌組織線溶活性の検討

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膀胱癌組織抽出液はフィブリン平板法において線溶活性を示さず, urokinase inhibitor の約 250 units/ml を含有した. ゲル濾過法 (Sephadex G-200) を用いて, この抽出液中に fibrinolysis activators と inhibitors の存在することが判明した. ヒト正常膀胱からの粗製 tissue activator はこの癌抽出液により活性が抑制された. urokinase は同じ抽出液により高度に抑制され, urokinase 30 Ploug units はこの抽出

液中に認められた urokinase inhibitor 250 units によりほとんど完全に抑制された. 一方この同じ抽出液の量により, tissue activator 8 Ploug units はその 30%のみが抑制されたにすぎなかった. 正常膀胱癌組織抽出液は plasminogen activator による中等度の線溶活性を示すとともに, urokinase に対してはきわめて軽度の抑制効果 (約 6 units/ml) を示した.